THE MECHANISM OF CLOT DISSOLUTION BY PLASMIN *

By NORMA ALKJAERSIG, ANTHONY P. FLETCHER and SOL SHERRY

(*From the Department of Medicine, Washington University School of Medicine, St. Louis, Mo.*)

Normal human and animal sera contain a globulin, plasminogen, which in the presence of activators is rapidly converted to plasmin, a proteolytic enzyme active at neutral hydrogen ion concentrations. Plasmin is an enzyme of wide specificity and will attack such varied substrates as gelatin, casein, certain synthetic esters, accelerator globulin, complement, fibrinogen and most importantly fibrin. Plasminogen activation occurs spontaneously (1), or as a result of contact with activators of tissue (2), body fluid (3, 4), or bacterial origin (5). The conversion of plasminogen to plasmin involves the loss of a peptide moiety and there is evidence to suggest that the plasmin obtained by different modes of activation may vary in composition (6).

Biochemically, considerable species differences exist not only between the plasminogen system of man and animals, but more particularly between the systems of various animals; this variability is most extreme with regard to the differential effectiveness of streptokinase. Rigid kinetic studies (6) reveal that the activation of human plasminogen under the influence of streptokinase (an extracellular product of hemolytic streptococcal metabolism), trypsin and urokinase (prepared from human urine) results from a first order enzymatic reaction.

Since physiological fibrinolytic phenomena result from activity of the plasminogen system, attempts to use plasmin or plasminogen activators to effect therapeutic thrombolysis have been numerous. Animal experiment, despite the difficulties and confusion of species variability, has yielded striking findings (7, 8, 9). Though the demonstration of experimental thrombolytic action has been of an unequivocal nature, the precise mechanism of its production has hitherto been obscure.

The present communication describes *in vitro* experiments and *in vivo* observations bearing upon thrombolytic [1] mechanisms in man. The results indicate that since plasminogen is found both in plasma and also as a constituent of thrombi, clot lysis occurs by a dual mechanism. The chief and primary mechanism of thrombolysis involves the diffusion or adsorption of plasminogen activator to the thrombus, activation of intrinsic clot plasminogen and thrombolysis. The secondary mechanism involving digestion of the thrombus by extrinsic plasmin action appears to be of little quantitative importance.

MATERIALS AND METHODS

Streptokinase [2] was a highly purified preparation, biophysically though not immunochemically homogenous; the specific activity was 600 to 700 streptokinase units per μg. nitrogen.

Human plasminogen (1) contained 100 to 150 casein units per mg. tyrosine.

Plasmin was prepared by autocatalytic activation of plasminogen in glycerol (1) and contained 95 casein units per mg. tyrosine (see Casein assay).

Epsilon amino caproic acid [2] (6-amino hexanoic acid) had a melting point of 208° C. and an elemental analysis corresponding in composition to that of the pure compound.

Urokinase [3] contained 5,100 Ploug units per mg. dry weight (10).

Fibrin plate test was modified from Müllertz (11) by using a highly purified fibrinogen preparation [4] in a final concentration of 0.2 per cent. *Heated fibrin plates* (12)

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[1] The term thrombolytic is used to designate a process by which preformed clots are lysed. This usage corresponds to the literal meaning and original derivation of the word (Oxford English Dictionary) and its use does not imply specific reference to pathological states where intravascular thrombi may be of variable composition.

[2] Kindly supplied by Dr. J. Ruegsegger, Lederle Laboratories, Pearl River, N. Y. Streptokinase activity expressed in Christensen units (J. clin. Invest. 1949, 28, 163).

[3] Kindly supplied by Dr. J. Ploug, Leo Pharmaceuticals, Copenhagen, Denmark.

[4] Bovine fibrinogen, salt free, 92 per cent of the nitrogen being clottable by thrombin; kindly supplied by Dr. Kent Miller, New York State Institute of Health, Albany, N. Y.

were prepared by subjecting fibrin plates to 80° C. for 30 minutes. This procedure resulted in denaturation of the contaminating plasminogen, rendered the plates insusceptible to the action of plasminogen activators, but also reduced their sensitivity to proteolysis (pure chymotrypsin [5] 0.05 mg. per ml. gave a zone of 473 mm.[2] on unheated plates and 276 mm.[2] on heated ones, while the figures for spontaneously activated plasmin (1.3 casein units per ml.) were 350 mm.[2] and 172 mm.[2], respectively).

Fibrinogen was estimated by the method of Ratnoff and Menzie (13) modified only by the addition of 0.1 ml. of 1 per cent soy bean trypsin inhibitor [5] to prevent further fibrinolysis.

I^{131} *labeled plasma clot test.* Fibrinogen [4] was iodinated by a method modified from that of Eisen and Keston (14). Assuming a molecular weight for fibrinogen of 350,000 (15), the degree of iodination varied between 0.6 and 1.3 I^{131} atoms for each molecule of fibrinogen. Free I^{131} was removed by passage through an Amberlite IRA-400 column (40 mesh) and integrity of the fibrinogen molecule demonstrated by the fact that after iodination treatment the nitrogen clottable by thrombin was 92 per cent (the starting value). Eighty-nine to 93 per cent of the radioactivity was clottable by thrombin.

Preformed isotopically labeled human clots were made by adding 1 to 4 λ of the iodinated fibrinogen (1 to 2 per cent solution) to 0.5 ml. of bulk plasma containing added plasminogen (*vide infra*) in an 8×80 mm. serological tube. The quantity of added radioactivity was calculated to give approximately 100 cpm per μg. of contained fibrinogen. For convenience, throughout the test, observed counts per minute have been mathematically transformed so that a single count per minute corresponds to the lysis of 0.8 μg. of fibrin. A wire, nichrome 26 S. W. G., coiled at its lower end, was inserted into each tube and 0.1 ml. of thrombin (50 units per ml.) was added. The clots were incubated for one to two hours in a 37° C. water bath and after retraction were withdrawn by means of the wire. Approximately 50 such clots were washed in 2 L. of normal saline overnight at 2° C. This procedure resulted in a low and consistent blank value for release of radioactivity.

I^{131} labeled plasminogen deficient, plasminogen normal and plasminogen rich clots

Plasminogen deficient clots. I^{131} labeled human plasma clots vigorously washed in a large excess of phosphate saline buffer (0.1 M) at pH 7.6 for 24 hours at 2° C. were shown to contain less than 0.15 casein units of plasminogen per clot (unwashed drained clots contained 0.3 to 1 casein unit). These clots were relatively resistant to lysis by plasminogen activators, while retaining a normal sensitivity to the action of plasmin. Residual plasminogen could be denatured by heating these clots in buffer at 80° C. for 30 minutes. Such clots, designated as "heated clots," were completely resistant to lysis by plasminogen activators but the heating process had

reduced their sensitivity to the action of plasmin so that it was approximately 60 per cent of that shown by unheated clots.

Clots of normal and high plasminogen content. Since clots had to be washed to reduce blank radioactivity values and this process reduced clot plasminogen, the preparation of clots of normal plasminogen content required reinforcement of the original plasma with purified plasminogen. This problem was simplified by the fact that while plasma plasminogen could be washed from plasma clots, purified plasminogen, owing to its different physical state of dispersion at pH 7.6, was completely taken up from the plasma into the clot and was not washed out. Plasma clots of normal plasminogen content were made by fortifying the original plasma with 0.5 to 1 casein unit plasminogen per clot. Plasminogen rich clots were made similarly except that the added plasminogen was 2 casein units plasminogen per clot. These latter clots were extremely sensitive to the presence of plasminogen activators and exhibited normal sensitivity to lysis by plasmin.

Thrombolytic [6] *assays* were made by inserting a prepared clot into 0.5 ml. of plasma or other specimen in a 10×100 mm. tube and incubating at 37° C. for 30 minutes. The clot was then withdrawn, removed from the wire and centrifuged in 2 ml. of normal saline; the supernatant was added to the residual plasma and the radioactivity determined in a well-type scintillation detector (efficiency 45 per cent). Comparison of residual clot nitrogen content with residual radioactivity demonstrated that clot lysis and radioactivity release were highly correlated.

Assay of isotopically labeled clots for plasminogen. Single clots were ground with powdered glass in a tube containing 0.5 ml. 1/6 N hydrochloric acid. After 15 minutes at room temperature, the supernatant solution was neutralized and assayed for plasminogen by the casein method. The validity of this assay procedure was tested on a series of 15 plasmas, to some of which had been added plasminogen and to others fibrinogen. The mean recovery of plasminogen from clot plus supernatant serum was 94 ± 9 per cent (range 79 to 109 per cent) of the original plasma aliquot.

Assay for plasminogen and plasmin in plasma

Casein assay. The casein assay (16) was modified from that of Remmert and Cohen (17), our unit being

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[5] Worthington Biochemical Corp., Freehold, N. J.

[6] The presence of plasma inhibitors requires that a clear distinction be drawn between total demonstrable activator or enzyme activity and that portion that is physiologically active (uncombined with inhibitor). Test systems requiring dilution or other changes in biophysical conditions are unacceptable for the assay of physiologically active components in the complex milieu of plasma, since these changes alter the ratios of combined to uncombined activities. Assay of these individual activities in undiluted plasma by means of isotopically labeled plasma clots of varying plasminogen concentration fulfills these stringent requirements.
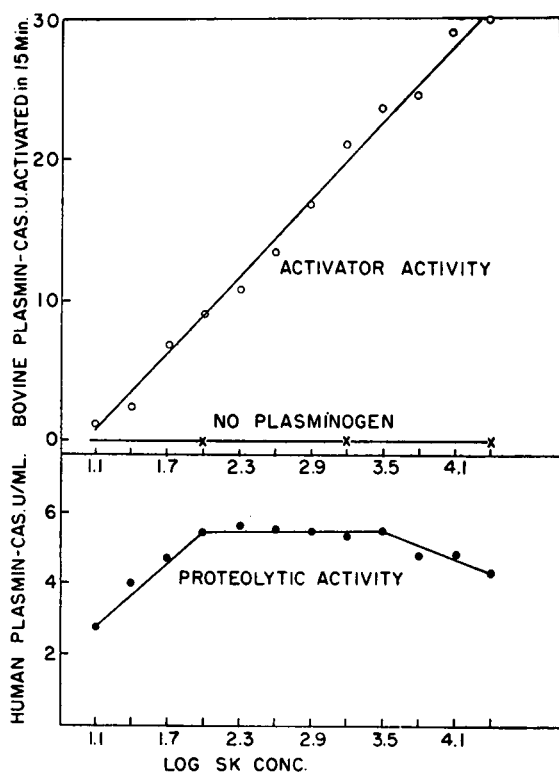
FIG. 1. ACTIVATOR AND PROTEOLYTIC ACTIVITIES OF HUMAN PLASMINOGEN AT VARIOUS STREPTOKINASE (SK) CONCENTRATIONS

Plasminogen concentration, 5.6 casein units per ml. Activator activity was approximately related to the logarithm of the streptokinase concentration (indicated on the figure as streptokinase log units per ml.) and was independent of the proteolytic activity.

Esterase activity was determined by the hydrolysis of benzoyl arginine methyl ester (BAMe) (18). The degree of hydrolysis was measured by the Hestrin colorimetric method (19). In contrast to the findings with casein plasminogen assay, prior acidification of the plasma was unnecessary if BAMe were used as a substrate. Though the reason for this discrepancy is uncertain,[8] the experimental difference has been adequately validated.

Plasminogen "activator" assay. The activation of plasminogen by streptokinase occurs as a two stage reaction (20, 21). Streptokinase first reacts with a plasma protein, termed proactivator,[9] to form activator and the activator converts plasminogen to plasmin by enzymatic transformation (20, 21). Since proactivator is absent from bovine plasma, bovine plasminogen may be used to assay "streptokinase activator" present in the plasma of other species.

Five-tenths ml. of bovine Fraction III_2 (Armour) (20 mg. per ml. or 5.6 casein units per ml.) was incubated for 15 minutes at room temperature with 0.1 ml. of the test solution. The activation reaction was then stopped by the addition of epsilon amino caproic acid to a final concentration of 0.08 M (16) and the solution assayed for plasmin activity by the casein assay. The results were expressed as casein units bovine plasminogen activated per 15 minutes.

RESULTS

The action of streptokinase on plasminogen preparations

Figure 1 illustrates activator and proteolytic activity assays made after the addition, 15 minutes previously, of increasing quantities of streptokinase to a purified human plasminogen preparation. It is evident that the two distinct activities of plasminogen preparations developed under the influence of streptokinase, the proteolytic and the activator activities, are differently related to the streptokinase concentrations employed. Whereas, proteolytic activity reaches a plateau at 100 streptokinase units per ml., which is maintained with

aproximately 50 per cent that of the latter authors. Assay of untreated plasma for plasminogen yielded low values and recovery of added plasminogen was incomplete owing to the presence of antiplasmin activity in untreated plasma. However, direct assay of plasma antiplasmin content revealed that this activity was largely or completely destroyed by adding 0.5 ml. of 1/6 N hydrochloric acid to 0.5 ml. of plasma, leaving at room temperature for 15 minutes and then neutralizing with 0.5 ml. of 1/6 N sodium hydroxide and 1 ml. of 0.1 M phosphate buffer at pH 7.6. With this modification recovery of added plasminogen or plasmin became quantitative and assay values for plasma plasminogen were substantially increased. All plasma used for casein assay was treated in this manner.[7]

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[7] The use of this method for the assay of plasmin in plasma may, under certain conditions, yield spuriously high values. There is evidence that, if the plasma contains both activator and plasminogen, the plasma manipulation (dilution, and so forth) consequent upon the assay procedure may facilitate plasminogen activation.

[8] Presumably the affinity of plasmin for BAMe is greater than for antiplasmin.

[9] There is still controversy as to whether proactivator is an entirely separate substance or is derived from plasminogen itself (22, 23, 24), but the term is used to designate a plasma factor capable of reacting with streptokinase to form plasminogen activator. Though divergent emphasis has been placed on the significance of proactivator in the human as apart from the bovine system (25, 26, 27) for descriptive purposes we acknowledge its separate existence and assay for its biochemical activity even though its individual identity apart from plasminogen or its derivatives has not been clearly established.
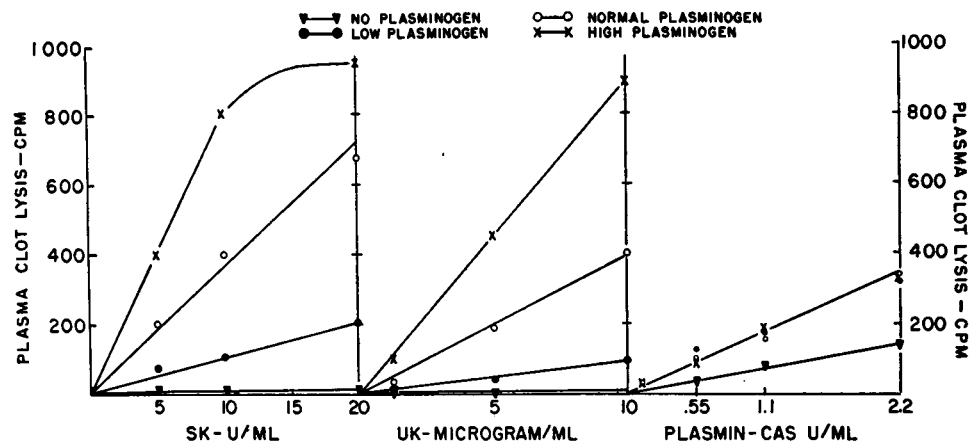
FIG. 2. THE LYSIS RATE OF I^{131} TAGGED PLASMA CLOTS AS A FUNCTION OF THE PLASMINOGEN
CONTENT OF THE CLOT AND OF THE ACTIVATOR AND PLASMIN CONCENTRATIONS

Lysis rates for streptokinase and urokinase varied as the logarithm of plasminogen concentration being zero in clots devoid of plasminogen. Lysis rates with plasmin were uninfluenced by the clot plasminogen concentration except that where clots were devoid of plasminogen, a lesser rate of lysis was found, due to denaturation caused by the heating process.

increasing streptokinase concentrations until it ultimately commences to decline around 3,000 streptokinase units per ml., activator activity behaves differently. Activator activity continues to increase with increasing streptokinase concentrations and is still rising at the highest streptokinase concentration used (25,000 streptokinase units per ml.). Since thrombi normally contain significant quantities of plasminogen, it is apparent that under the influence of streptokinase, plasminogen preparations develop two types of activities, proteolytic and activator, either of which is potentially capable of mediating the lysis of a thrombus.

The effect of streptokinase, urokinase and plasmin upon plasma clots

Figure 2 shows the rate of supernatant radioactivity release determined when isotopically labeled plasma clots of differing plasminogen concentration (four concentrations displayed in each panel) were incubated at 37° C. for 30 minutes with several concentrations of streptokinase, urokinase and spontaneously activated human plasmin (in 0.1 M phosphate buffer at pH 7.6). The assay technique was that described under the heading of thrombolytic assay in the Methods section.

The results for streptokinase and urokinase (Panels 1 and 2) show that not only is the rate of clot lysis linearly related to the surrounding

activator concentration,[10] but that it is also, at any given activator concentration, related, though not directly proportional, to the clot plasminogen concentration. In fact, in this and other experiments, over a "physiological" range of clot plasminogen concentrations, clot lysis rates at a given activator concentration have been approximately proportional to the logarithm of the clot plasminogen concentration. It is evident that clots lysing in a streptokinase solution do so because there are ample amounts of proactivator as well as plasminogen in the clot.

Panel 3 illustrates the effect of spontaneously activated human plasmin. The lysis rate rises linearly with enzyme concentration but the release of radioactivity was independent of clot plasminogen concentration. Though clots of high, normal and low plasminogen concentration were lysed at identical rates, the lysis rates of those clots marked "no plasminogen" were about half those of the others. This occurred as a consequence of their method of preparation, which involved heating, and the diminished sensitivity to plasmin action was similar to that recorded with the heated fibrin plates (see Methods section). The sensitivity of the unheated clots to the action of plasmin in

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[10] This relationship should not be interpreted as favoring either of the two alternative views concerning passive diffusion of or active absorption of streptokinase or urokinase to a clot.

terms of fibrin lysis rates was one-fifth to one-tenth that shown by purified fibrinogen or purified fibrin in a diffuse state. Whether this diminished sensitivity was due to the relative impurity of the fibrin in the washed clot or due to the mechanical barrier to enzyme penetration posed by the structure of the clot is uncertain, but the findings do suggest that digestion of thrombi, *in vivo*, by extrinsic plasmin action may be somewhat impeded.

Thrombolytic activity in plasma

The results of adding an excess of streptokinase to plasma are illustrated in Figure 3. Seventy streptokinase units were added per ml. of plasma, but since the antibody content of the plasma was equivalent to 30 streptokinase units per ml., the results illustrated refer to the presence of 40 free streptokinase units per ml. plasma. The upper portion of the figure shows that rapid activation

FIG. 4. THE DEPENDENCE OF STREPTOKINASE EFFECT UPON CLOT PLASMINOGEN CONCENTRATION

Clots devoid of plasminogen were unaffected by streptokinase treated plasma. Clots containing plasminogen were lysed by streptokinase treated plasma at rates dependent upon their plasminogen concentration.
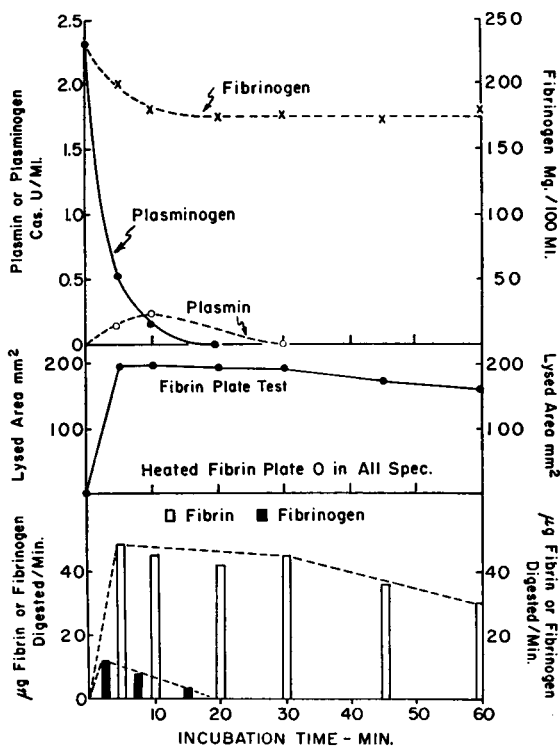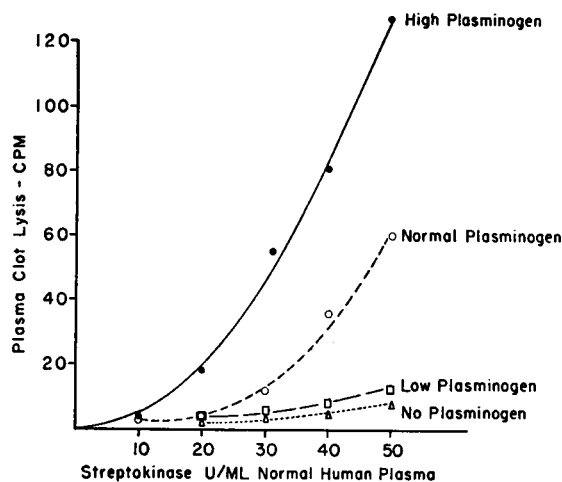

FIG. 3. BIOCHEMICAL CHANGES PRODUCED BY STREPTOKINASE IN PLASMA

Seventy streptokinase units were added per ml. plasma at zero time. The plasma activity developed was primarily thrombolytic in nature and fibrinogenolysis was minimal. This comparison is most strikingly seen in the bottom section of the figure.

of plasminogen occurred, so that at the end of 10 minutes only 10 per cent of the original quantity remained and after 20 minutes a zero assay reading was obtained. Concomitantly with the fall of plasminogen concentration, plasmin activity of low degree was detected, but it is noteworthy that this activity was only detectable after plasma antiplasmin activity had been destroyed by acidification (see Methods section). Assay of unacidified plasma for plasmin activity (not illustrated in Figure 2) revealed the presence of little or no proteolytic activity.

Fibrinogen levels declined to 75 per cent of their original value and then became stable; these assay values have been calculated as rates of fibrinogen lysis (μg. lysed per minute) and are plotted as such in the lower portion of Figure 3. However, in contrast to the low degree and short duration of plasma fibrinogenolytic activity, assay for plasma thrombolytic activity by means of the isotopically labeled clot technique (using clots of normal plasminogen content) disclosed an intense and sustained state of clot lysing activity. This activity, calculated as rates of fibrin lysis (μg. clot fibrin lysed per minute), has been plotted in the lower portion of Figure 3, where it may be compared with the rate of fibrinogen lysis calculated similarly. Comparison of these biochemical activities shows extreme divergence

between their magnitudes and durations, plasma clot lysing power being predominant in both respects. The fibrin plate assay results, displayed in the middle portion of Figure 3, confirm this conclusion. Assay on unheated fibrin plates showed a high and sustained degree of plasma thrombolytic activity that paralleled that revealed by the isotopic clot dissolution test. However, all readings made on heated fibrin plates were zero, indicating a plasmin activity of less than 0.15 casein unit per ml. (sensitivity limit of the method).

The biochemical findings illustrated in Figure 3 are typical of those found on addition of streptokinase to plasma. Variation of the streptokinase concentration, at least within the range of five to 50 free streptokinase units per ml. plasma, while altering the speed and degree of the reaction does not influence its general qualitative nature.

The relationship of thrombolysis, plasma thrombolytic activity and clot plasminogen concentration

Figure 4 shows the degree of lysis produced in clots of differing plasminogen content immersed in plasma containing varying concentrations of streptokinase. The four clot plasminogen concentrations tested were those used in the experiment shown in Figure 2 and the plasma antibody content was equivalent to 15 streptokinase units per ml. Lysis failed to occur in any of the clots immersed in plasma containing 10 streptokinase units per ml. and it has been an invariable finding that significant thrombolysis has always been absent unless the quantity of added streptokinase has been sufficient to exceed the antibody binding power of the plasma. Though under special circumstances using different techniques a limited dissociation of the streptokinase antibody complex can be demonstrated, provided that the system is not diluted, dissociation of the streptokinase antibody complex exerts no practical effect.

The assays conducted at streptokinase concentrations of 20, 30, 40 and 50 units per ml. plasma showed an increasing divergence between the lysis rates of clots containing different plasminogen concentrations. Where clot plasminogen was low or absent, thrombolysis was negligible or absent. In contrast, clots containing a normal plasminogen concentration were rapidly lysed and this action

was greatly enhanced if plasminogen rich clots were used. Thrombolysis rates with clots of a given plasminogen concentration were approximately linear with increasing concentrations of free streptokinase and the somewhat concave curves shown in Figure 4 probably resulted as a consequence of the varying time relations of reactions in the complex matrix of plasma. As in the earlier experiment shown in Figure 2, the rates of clot lysis, at any given activator concentration, were approximately related to the logarithm of the clot plasminogen concentration, though the data fitted this relationship far less exactly than in the former case.

These experimental findings, which closely mimic those found in vivo, demonstrate that clot lysis, at a given activator concentration, is dependent upon the plasminogen content of the clot. A corollary to these findings indicates that thrombolysis, at least under physiological conditions, must be dependent upon activation of intrinsic clot plasminogen. Moreover, the illustration that plasminogen deficient clots are lysed poorly, if at all, by streptokinase activated plasma speaks strongly against the concept that thrombolysis is related to extrinsic plasmin action under physiological circumstances.

The influence of an inhibitor of plasminogen activation upon thrombolytic activity displayed by streptokinase activated plasma

Plasminogen activation, in the presence of plasminogen activators, is inhibited competitively by low concentrations of epsilon amino caproic acid, themselves insufficient to inhibit plasmin action (16). Figure 5 (lower portion) displays such evidence, obtained by the fibrin plate method and pertaining to the experimental conditions used in the upper portion of Figure 5. The lower part of the figure shows that the high assay reading produced by plasma containing 50 streptokinase units per ml. was completely abolished by the addition to the mixture, prior to assay, of 0.02 M epsilon amino caproic acid. The action of plasmin on the plate, adjusted in amount so as to yield a zone of lysis comparable with that of the streptokinase activated plasma, was virtually unaffected by the addition of 0.02 M epsilon amino caproic acid.
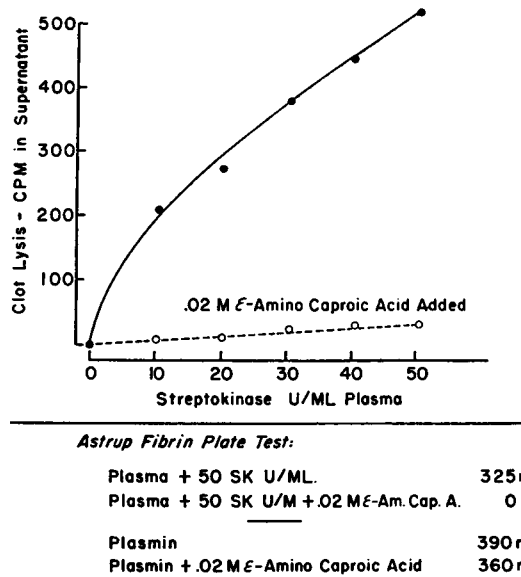
Astrup Fibrin Plate Test:

Plasma + 50 SK U/ML.	325 mm²
Plasma + 50 SK U/M +.02 ME-Am.Cap. A.	0
Plasmin	390 mm²
Plasmin +.02 M E-Amino Caproic Acid	360 mm²

FIG. 5. EFFECT OF ε-AMINO CAPROIC ACID ON PLASMINOGEN ACTIVATOR (MEASURED ON PLASMINOGEN RICH PLASMA CLOTS)

The bottom portion of the figure demonstrates that ε-amino caproic acid added to streptokinase treated plasma in a concentration of 0.02 M acts solely to inhibit plasminogen activation. The top portion of the figure demonstrates the abolition of thrombolytic activity in streptokinase treated plasma to which ε-amino caproic acid has been added.

The upper portion of Figure 5 shows that the powerful thrombolytic action of plasma containing varying concentrations of streptokinase on plasminogen enriched clots was totally suppressed by the addition of 0.02 M epsilon amino caproic acid. Though plasminogen enriched clots were used for this experiment precisely similar results have been obtained when clots containing different plasminogen concentrations were used. These findings offer powerful support to the concept that plasma thrombolytic activity is related to its content of plasminogen activator and that free plasmin activity plays little or no part in thrombolysis.

Evidence from in vivo experiment

In vitro experiments, such as have been described here, may not be directly applicable to the *in vivo* state, since changes in plasma composition may alter the experimental conditions. The biochemical changes occurring in man as a result of massive and prolonged streptokinase infusion have been described elsewhere (24), but

two problems, relevant to the application of the present findings to *in vivo* circumstances, are considered in this communication.

Plasmin assays made in 17 patients (total of 88 plasma samples), receiving streptokinase therapy, revealed no evidence of greater plasmin release *in vivo* than would have been anticipated from *in vitro* plasma assay. Sufficient streptokinase was infused to activate all the plasma plasminogen within a one to two hour period and then maintain the plasminogen concentration at zero for the remainder of the treatment period. Plasmin activity was only detectable for the first two to three hours of the treatment period and peak values in the individual patient were of short duration. Assay values found were always less than 10 per cent and usually less than 5 per cent of the potential activity (total conversion of plasminogen to plasmin would equal 100 per cent). Indeed it is possible that these values may have been even lower, as the plasma manipulations, required for assay purposes, may have resulted in a spurious increase in free plasmin assay values.

Figure 6 illustrates the effect of the various fibrinolytic moieties found in plasma during streptokinase therapy upon an assay system of clots
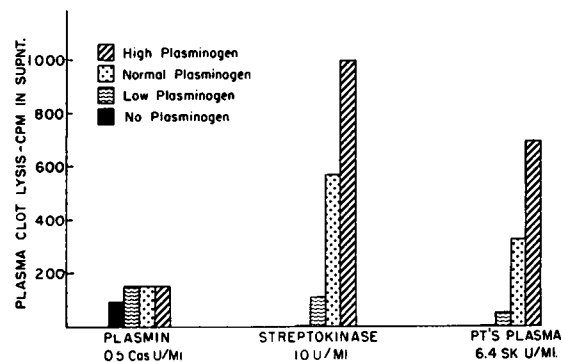

FIG. 6. EFFECT OF FIBRINOLYTIC MOIETIES PRESENT IN STREPTOKINASE (SK) ACTIVATED PLASMA UPON RADIOACTIVE CLOTS

The right hand column shows the thrombolytic activity of plasma drawn from a patient receiving streptokinase therapy upon clots of different plasminogen content. The general similarity of this pattern of activity to that displayed in the center column (the action of streptokinase upon identical clots) and its dissimilarity to the pattern of activity found with plasmin (shown on the left) is obvious. Furthermore the quantity of plasmin used was much greater than can ever be demonstrated in plasma.

containing four different plasminogen concentrations. On the left is shown the effect of the maximal plasmin activities found *in vivo* (after acidification of the plasma); in the center, the effect of streptokinase (10 units per ml. buffer) on the same assay system; and on the right, the effect of a plasma sample withdrawn from a patient three hours after the start of treatment. This patient received tracer doses of isotopically labeled streptokinase for the purpose of determining plasma streptokinase concentrations and at the time that the sample was drawn the value for free streptokinase units per ml. plasma was 6.4. The center and right hand columns show identical ratios of activity on clots of different plasminogen content and it is apparent that *in vivo* and *in vitro* clot lysis mechanisms are similar in nature.

DISCUSSION

Human plasminogen preparations, when treated with streptokinase, develop both activator and proteolytic activity. However a marked dissociation is apparent between the streptokinase/plasminogen ratios required for the maximal development of these two activities. With a fixed quantity of plasminogen, the addition of a relatively limited amount of streptokinase will induce full proteolytic activity but a restricted degree of activator activity. Increasing concentrations of streptokinase cause an increase of activator activity approximately related to the log concentrations of the added streptokinase, but proteolytic activity does not increase and later, when activator activity is still rising, commences to decline.

In a like manner, the addition of an excess of streptokinase to plasma produces both a proteolytic and also an activator effect. The proteolytic activity is almost completely inhibited by plasma antiplasmin, but the activator effect (assayed on the bovine fibrin plate, which is not sensitive to the action of streptokinase alone) persists, even when plasminogen is no longer demonstrable as a consequence of its activation. These findings are identical with those obtained by study in patients, in whom activator formation remained unimpaired after plasma plasminogen concentrations had been maintained at zero or virtually zero levels for 30 hours by streptokinase infusion (24).

Though plasminogen activation occurs as a two stage process (20, 21) and requires proacti-

vator as an essential component for its completion, clots containing plasminogen also contain a sufficient amount of proactivator to allow of their lysis by streptokinase alone. Experiment showed that clots contained in streptokinase buffer solution lysed in a manner comparable with clots suspended in streptokinase treated plasma. Thus the lysis of clots in streptokinase treated plasma, by activation of their contained plasminogen, could occur either through the direct action of preformed activator or be caused indirectly by streptokinase alone. Insufficient evidence is available to evaluate the relative importance of these mechanisms, but conceivably, under *in vivo* conditions, one may be more important than another.

These results offer no evidence to support the view that digestion of clots or thrombi by the direct action of circulating plasmin is of practical importance. First, the appearance of plasmin, during and after the process of complete activation of plasminogen *in vitro*, is transient and its concentration is very low. Second, assay of plasma from patients receiving streptokinase therapy has shown that no greater quantity of plasmin is formed *in vivo* than would have been expected from predictions based on *in vitro* plasma assay. Third, *in vitro* experiment showed that plasmin digested clots, of differing plasminogen content, at a uniform, relatively slow rate and that if used in concentrations similar to those found in plasma its effects were negligible. Fourth, plasma drawn from patients, receiving streptokinase therapy, lyses clots of varying plasminogen content in a manner identical to that of plasma to which streptokinase has been added *in vitro* and differently from that seen with plasmin. Lastly, the addition of a specific inhibitor of activator, epsilon amino caproic acid (16), in a concentration insufficient to inhibit plasmin, abolishes plasma thrombolytic activity.

On the other hand, there is strong evidence that thrombolysis results from the direct or indirect activation of thrombus plasminogen. First, direct assay for plasminogen, within the thrombus, disclosed the presence of sufficient plasminogen to account for the observed results. Second, with a fixed activator concentration contained in either buffer or plasma, thrombolysis rates were linearly related to the logarithm of the clot plasminogen concentration. Third, with clots of fixed plas-

minogen concentration, thrombolysis rates were linearly related to the logarithm of the activator concentration. Fourth, assays for activator were highly correlated with thrombolysis rates of clots containing normal amounts of plasminogen. Lastly, identical patterns of thrombolysis were produced by comparable plasma specimens whether these were prepared *in vitro* or drawn from streptokinase treated patients and in both cases low concentrations of epsilon amino caproic acid completely inhibited thrombolytic activity.

These experiments demonstrate that the chief and primary mechanism of plasma thrombolysis involves the activation of intrinsic clot plasminogen with resulting autodigestion of the thrombus and that the secondary mechanism of thrombolysis by exogenous plasmin action appears to be relatively unimportant. Therefore we propose that the word thrombolysis be used to designate the entire phenomenon by which preformed thrombi are lysed and which for practical purposes describes a complex series of events inclusive of a final step involving the local action of plasmin confined within the substance of the thrombus. The word fibrinolysis will then be restricted to its original meaning and will designate the action of a proteolytic enzyme acting primarily upon a fibrin substrate, but also acting secondarily upon such other substrates as are susceptible to its action.

The necessity for such a clarification of nomenclature is emphasized by the finding that the addition of urokinase to plasma produces similar results to those reported with streptokinase; a finding that also serves to establish the general importance of these mechanisms to the body economy. These observations exemplify a new biological principle of perhaps considerable significance as they constitute the first demonstration of how an enzyme of wide proteolytic activity (plasmin) may *in vivo* retain its full spectrum of activity, but through local mechanisms have its specificity virtually limited to one substrate (fibrin). Indeed, unless such a mechanism existed, phenomena causing plasminogen activation might well be attended by catastrophic consequences to the organism.

SUMMARY

1. The respective roles of plasminogen activators and plasmin in human thrombolytic mecha-

nisms have been investigated. Quantitation of thrombolysis was accomplished by the use of I^{131} labeled human plasma clots. Test systems using labeled clots containing varying concentrations of plasminogen permitted differentiation between the actions of plasminogen activators and plasmin itself.

2. Studies on streptokinase treated patients confirmed that the conclusions drawn from *in vitro* test systems were applicable to the *in vivo* state.

3. It was demonstrated that the chief and primary mechanism of plasma thrombolysis involved the activation of intrinsic clot plasminogen with resulting autodigestion of the thrombus. The secondary mechanism of thrombolysis by exogenous plasmin action appeared to be relatively unimportant.

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